Life Sciences in Space Research 6 (2015) 59–68

Contents lists available at ScienceDirect

Life Sciences in Space Research

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Developmental, nutritional and hormonal anomalies of weightlessness-grown wheat

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ARTICLE INFO

Article history:
Received 5 May 2015
Received in revised form 8 July 2015
Accepted 9 July 2015

Keywords:
Ethylene
Hyperhydricity
Orbital space flight
Oxidative stress
Tillering
Triticum aestivum L

ABSTRACT

The behavior of water in weightlessness, as occurs in orbiting spacecraft, presents multiple challenges for plant growth. Soils remain saturated, impeding aeration, and leaf surfaces remain wet, impeding gas exchange. Herein we report developmental and biochemical anomalies of "Super Dwarf" wheat (*Triticum aestivum* L.) grown aboard Space Station Mir during the 1996–97 "Greenhouse 2" experiment. Leaves of Mir-grown wheat were hyperhydric, senesced precociously and accumulated aromatic and branched-chain amino acids typical of tissues experiencing oxidative stress. The highest levels of stress-specific amino acids occurred in precociously-senescing leaves. Our results suggest that the leaf ventilation system of the Svet Greenhouse failed to remove sufficient boundary layer water, thus leading to poor gas exchange and onset of oxidative stress. As oxidative stress in plants has been observed in recent space-flight experiments, we recommend that percentage water content in apoplast free-spaces of leaves be used to evaluate leaf ventilation effectiveness. Mir-grown plants also tillered excessively. Crowns and culms of these plants contained low levels of abscisic acid but high levels of cytokinins. High ethylene levels may have suppressed abscisic acid synthesis, thus permitting cytokinins to accumulate and tillering to occur.

1. Introduction

Crops have been grown successfully from seed to seed in weightlessness when root zone and canopy environments were appropriately modified (Sychev et al., 2001, 2007; Musgrave and Kuang, 2003). Nevertheless, studies suggest that weightlessness negatively impacts various physiological and developmental processes required for high yields (Musgrave et al., 2000, 2005; Ferl et al., 2002; Wolverton and Kiss, 2009; Paul et al., 2013; De Micco et al., 2014). The extent to which growth and yield are affected negatively by weightlessness, rather than by terrestrial stresses that are merely weightlessness-aggravated, is unclear (Wolff et al., 2013; De Micco et al., 2014). Solving this question will require additional hardware experiments wherein weightlessness-aggravated terrestrial stresses are identified and minimized. Developmental anomalies persisting after such iterations of experimentation may indeed have weightlessness-based etiologies.

In 1996–97, two experiments were conducted aboard the Russian Space Station Mir using 'Super Dwarf' wheat (*Triticum aestivum* L.). For the first experiment, plants were grown for 123 d. Compared to Earth-grown controls, the Mir-grown plants were shorter, lighter and had tillered excessively. They had also produced floral spikes, but no seeds (Levinskikh et al., 2000). Gas analysis measurements aboard Mir indicated that concentrations of ethylene, a gaseous plant hormone, were consistently above physiologically-detrimental levels. From subsequent trials on Earth, where ethylene levels were tested, it was concluded that ethylene had caused the observed growth anomalies and the failure of seeds to form (Campbell et al., 2001).

Following harvest of the 123-d experiment, the root modules were reseeded to produce a second set of Super Dwarf plants. Thirteen days after sowing, leaf bags were placed over the canopies, and for 12 d photosynthesis and transpiration rates were measured (Monje et al., 2000). After an additional 16 d (41 d total), the plants were harvested, and the shoots and some roots were frozen and transported to Earth for morphological, elemental, hormonal and free amino acid analyses. Data obtained from these analyses and analyses of reference plants grown under favorable conditions on earth were summarized in NASA reports. However,

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http://dx.doi.org/10.1016/j.lssr.2015.07.005

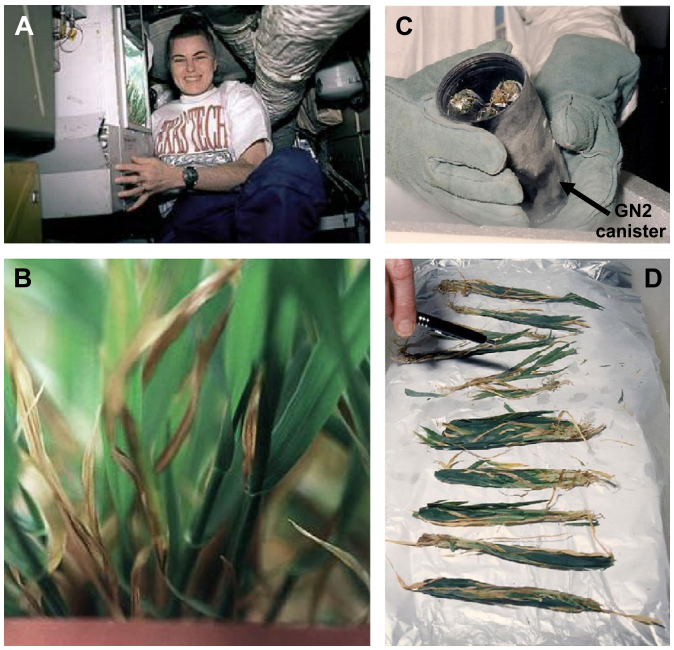

Fig. 1. Greenhouse-2 wheat growing in the Svet growth chamber aboard Mir (123-d experiment, A–B) and frozen wheat (41-d experiment, C–D) 3 h after Space Shuttle Atlantis (STS-81) landed at the Kennedy Space Center. (A) Astronaut Shannon Lucid displaying Svet. (B) Close-up of wheat in Svet. (C) Opened GN2 canister with aluminum-foil-wrapped wheat plants. The plants had been folded to fit in the canister. (D) Frozen wheat plants immediately after being unwrapped. Note early senescence of leaves (compare with B).

mock-up ground controls and analyses thereof were not funded. Consequently, the obtained data were not published.

Recent findings concerning plant metabolism and hormone physiology, and recent reports of oxidative stress coupled with excessive reactive oxygen species (ROS) formation in weightlessness-grown plants, have caused us to reconsider the publishing of our data. Of interest, our data provide evidence that the Mir-grown plants experienced hyperhydricity-induced anoxia followed by oxidative stress, autophagy and precocious leaf senescence. It has been postulated that elevated oxidative stress in weightlessness-grown plants is caused by radiation and unexplained weightlessness effects (Shagimardanova et al., 2010; Sugimoto et al., 2014). However, in our experiments major causes of oxidative stress appear to have been high ethylene levels and suboptimal leaf ventilation.

2. Materials and methods

2.1. Plant materials and growth conditions

Plants of the hexaploid wheat cv. Super Dwarf, CIMMYT selection CMH79.481-1Y8B-2Y-2B-OY (Salisbury et al., 1998), were grown aboard Mir in the Svet growth chamber as part of the 1996–97 Greenhouse-2 project (Bingham et al., 1996b; Campbell et al., 2001; Salisbury et al., 2003). Briefly, Svet consisted of two Balkanine-filled root modules (0.1 m^2 canopy area) that supported up to 40-cm-tall plants. Balkanine is a clinoptilolite zeolite (Mumpton, 1999) from the Beli Plast deposit, Bulgaria, that is naturally charged with enough minerals for several cycles of plant growth (Table 1; Ivanova et al., 1997). Lighting in Svet was provided by fluorescent lamps that delivered a mean photosynthetic photon flux of 400 µmole m^{-2} s^{-1} (20/4 d/n photoperiod), and d/n temperatures were set for 28/21°C. Leaf ventilation was achieved by a cooling fan above the light banks, which drew air in at the base of the greenhouse and moved it out into the Mir cabin (Fig. 1A). Air flow through Svet occurred at 1500 L min^{-1}.

Table 1
Growth parameters for Mir-grown and reference plants.

Parameter	Mir-grown plants	Reference plants
Air temperature, *d*/*n* (C)	28/21[a]	22/16
Photoperiod, *d*/*n*	20/4[b]	16/8
Photosynthetically active photon flux, µmol m^{-2} s^{-1}	450[b]	500–700
CO_2, mmol mol^{-1}	7.5–9.1[b]	0.37
Ethylene, µmol mol^{-1}	1.1–1.7[c]	<0.1
Relative humidity, %	68–96[b]	20–30
Weightlessness	Yes	No
Soil substrate	Balkanine[a]	3:1:1[d]
Substrate moisture level, percent of saturation	40–70[a]	40–60
Nutrients[e], mg L^{-1}		
N		
NH_4	941	2.42
NO_3		3.78
Urea		6.20
P	88	5.42
K	20 126	10.33
Ca	6892	
Na	5152	
Mg	608	0.03100
Mn		0.01550
Fe		0.03100
B	5	0.00422
Cu	29	0.00223
Zn	101	0.00155
Mo		0.00062
Ag^+	0.2[b]	

[a] Levinskikh et al. (2000).
[b] Monje et al. (2000).
[c] Bingham et al. (1996a), James et al. (1997).
[d] Sunshine Mix #1, peat moss, sandy loam soil.
[e] Nutrients for Mir-grown plants are reported as PPM Balkanine (Ivanova et al., 1997) adjusted for bulk density, 0.84 (Kostov and Sapunova, 2009), and water content, 0.5 cm^3/cm^3 (Jones and Or, 1999). These values do not reflect actual levels of nutrient availability, which involve highly-variable dissolution and ion exchange rates. Nutrients available to reference plants were provided by Peters 20:20:20 fertilizer (250 mg L^{-1}) and Sunshine #1 Mix (equivalent to one full-nutrient application, not included).

Two crops of wheat were produced in the Svet greenhouse for the 1996–97 Greenhouse-2 experiments. The first crop was planted by astronaut Shannon Lucid on Aug 5, 1996 (Fig. 1A–B). It consisted of four rows of 26 seeds each, two rows in each of two root modules (104 seeds total). These were grown for 123 d. The plants were then harvested by astronaut John Blaha on Dec 6, 1996. Blaha reseeded the root modules on the same day. The second crop was grown for 41 d. The plants, consisting of shoots and some roots, were individually harvested by Blaha in January, 1997. The process included folding the plants, wrapping the plants in aluminum foil, and inserting the wrapped plants in the liquid-N_2-cooled GN_2 freezer (Fig. 1C). The plants were then transported to Earth aboard Space Shuttle Atlantis (STS-81 Mission) (Salisbury, 1997; Salisbury et al., 2003). Within 3 h of landing at the Kennedy Space Center (January 22, 1997), the frozen plants (Fig. 1D) were divided randomly into two groups, one for analyses at Utah State University (USU) and one for analyses at the Institute of Biomedical Problems, Moscow, Russia. Plants were shipped on dry ice to these locations. The present paper deals with the 18 41-d-old Mir-grown plants shipped to USU and with 20 additional Super Dwarf plants grown at USU under favorable conditions and referred to herein as reference plants.

A formal ground control experiment for the 41-d plants (2nd planting) using a Svet mock-up was not funded. Since rigorous simulation of multiple environmental variables experienced within Svet (Table 1) was not possible, we decided to produce a healthy, low-cost “reference” set of Super Dwarf wheat, to be grown under more ideal nutrient, lighting and temperature conditions, so as to

provide points of reference for interpreting the morphological and chemical anomalies observed among specific tissue types of the Mir-grown plants. The reference plants were grown in 1997 (post-flight) for the same period of time, 41 d, in a Perceval (Perry, IA) growth chamber in 3.7 L pots containing a 3:1:1 mixture of Sunshine Mix #1 (Sun Gro Horticulture Canada Ltd, Vancouver, BC, Canada), peat moss and sandy-loam soil. Light (photosynthetic photon flux of 500–700 $\mu mol\, m^{-2}\, s^{-1}$ at the canopy surface) was provided by fluorescent and incandescent lights using a 16/8 d/n photoperiod and a 22/16 °C d/n temperature regime. Plants were watered regularly with a dilute solution (250 $mg\, L^{-1}$) of Peters Professional 20–20–20 fertilizer (Scotts, Marysville, Ohio), which provided nutrients as listed in Table 1. All analyses of harvested plant materials, from Mir-grown and reference plants, were completed in 1997.

2.2. Morphometric analyses

The Mir-grown plants were separated on ice at USU according to numbers of tillers per plant, and the following variables were quantified: *i*) numbers of primary, secondary, and tertiary tillers per plant, *ii*) numbers of living and senescent leaves for each parent shoot and tiller, and *iii*) lengths and widths of the youngest fully-expanded leaf (sheath plus blade) for each parent shoot and tiller. Additionally, lengths and widths of all leaves were obtained for six randomly selected plants. Primary tillers are defined herein as tillers arising from the crown of the parent shoot (original shoot produced by the seedling). Secondary and tertiary tillers are defined as tillers arising from crowns of primary and secondary tillers, respectively. Leaf measurements were also made on tillers of the reference plants. Leaf areas of all plants were estimated according to the equation 0.75 × leaf length × leaf width (Sestak et al., 1971). The plants were kept frozen during morphometric analyses.

2.3. Tissue water measurements

Following morphometric analyses, Mir-grown plants were dissected on ice into tissue categories: roots, crowns, culms, young leaves, mature leaves and senescent leaves. Tissue samples from each individual parent shoot or tiller were kept separate from those of other parent shoots or tillers, and each was labeled such that it could be traced back to a given plant number, which identified the root module in which the plant was grown and the row number and location within the row. The reference plants were dissected and divided into the same tissue categories. Fresh mass (FM) values were obtained for each sample, the samples were lyophilized in pre-weighed micro-centrifuge tubes using a Savant Speedvac (Thermo Fisher Scientific, Inc., Waltham, MA), dry mass (DM) values were then obtained, and samples were stored at −80 °C until analysis.

Mir-grown samples were further partitioned according to dry mass. This was done by *i*) summing the DM of all leaves (young, mature, and old) of each parent shoot or tiller, *ii*) ranking the parent shoots or tillers by total leaf DM, and *iii*) partitioning them into high or low DM categories (Fig. 2). The cutoff between high and low DM categories was chosen near the median, i.e., so that near-equal quantities of DM were present in each category.

2.4. Mineral analyses

Lyophilized samples targeted for mineral analyses (Table 2) were digested using nitric and perchloric acids (Jones et al., 1991) and analyzed by inductively-coupled plasma spectrometry (Thermo Jarrell, Model ICAP 9000, Franklin, MA).

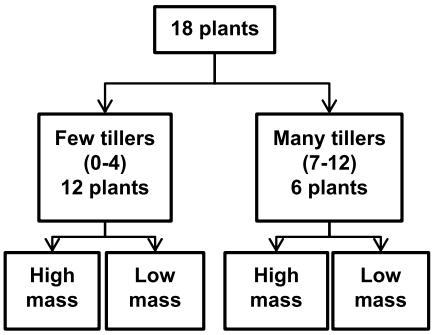

Fig. 2. Sampling of Mir-grown plants. The strategy was based on *i*) numbers of tillers produced per plant (few or many), and *ii*) size of parent shoots or tillers as determined by leaf DM (high or low). Most Mir-grown plants were dimorphic in terms of possessing both large and small tillers.

2.5. Analyses of free amino acids

Lyophilized samples targeted for free amino acid analyses (Table 2) were homogenized using a Brinkmann Polytron homogenizer (Metrohm USA, Inc., Riverview, FL) on ice in 5 mL tubes for 15–60 s at medium to high speed using a ratio of 15 mg sample to 1.0 mL high performance amino acid dilution buffer (Beckman 7300/6300, Beckman Coulter, Brea, CA) diluted 50 % (v/v) with deionized water. Samples were passed through a 0.2 µm filter and collected in a second 5 mL tube. The homogenizer bit was rinsed once using diluted buffer (same volume for the rinsate as was used for the initial grinding), and the rinsate was passed through the 0.2 µm filter. Samples were then dried in a rotary evaporator and brought to volume using dilution buffer: 15 mg sample to 1.0 mL dilution buffer. Vortexing and sonication were used to dissolve the pellet. Sample solutions were frozen until analysis. Sample aliquots (20 µL) were injected in a Beckman 6300 High Performance Amino Acid Analyzer, and data were electronically captured and analyzed. These procedures do not discriminate between glutamine and glutamic acid or asparagine and aspartic acid.

2.6. Hormone analyses

Levels of abscisic acid (ABA), indole-3-acedic acid (IAA) and six cytokinins (zeatin, zeatin riboside, dihydrozeatin, dihydrozeatin riboside, isopentenyl adenine, and isopentenyl adenosine) were analyzed as previously described (Hess and Carman, 1993, 1998; Nan et al., 1999). Briefly, samples were homogenized and lyophilized in methanol and spiked with tritiated hormones. Extracted hormones were purified using solid phase chromatography and HPLC, and HPLC fractions were collected along with aliquots for scintillation counting. Pooled hormones were lyophilized to dryness and were quantified by non-competitive indirect ELISA.

2.7. Statistical analyses

Descriptive statistics, analysis of variance with Tukey multiple range tests, and linear regression analyses were performed using SYSTAT Software (2004). Tests of significance were performed at the $P \leq 0.05$ level.

3. Results and discussion

The reference plant environment differed from that of the Svet environment in many ways including lighting, temperature, CO_2 and ethylene levels, relative humidity, soil properties, nutrient availability, and weightlessness (Table 1). Collectively, these factors caused Mir-grown plants and reference plants to develop

Table 2
Dry mass (DM), water content (H_2O %) and chemical analyses performed for tissue samples of Mir-grown plants and reference plants. Samples were partitioned based on numbers of tillers produced per plant (tillers per plant) and total shoot or tiller DM.

Sample[a]	Growth environment	Tillers per plant	Tiller or shoot DM	DM (mg)	H_2O (%)	Analysis[b]
R1	Mir	Few/many	High/low	65	86.0	H
R2	Mir	Few/many	High/low	69	84.3	H
K1	Mir	Many	High/low	127	84.4	H
K2	Mir	Many	High/low	122	84.8	H
K3	Mir	Few	High/low	122	85.4	H
K4	Mir	Few	High/low	100	85.5	H
C1	Mir	Many	High/low	85	83.8	H
C2	Mir	Many	High/low	132	85.3	H
C3	Mir	Few	High/low	66	85.0	H
C4	Mir	Few	High/low	52	87.4	H
Y1	Mir	All	High/low	87	85.6	H
Y2	Mir	All	High/low	83	86.3	H
M1	Mir	Many	High	712	82.5	M, AA, H
M2	Mir	Many	High	601	77.1	M, AA, H
M3	Mir	Many	Low	510	84.3	M, AA, H
M4	Mir	Many	Low	529	82.6	M, AA, H
M5	Mir	Few	High	402	82.9	M (5 + 7), AA, H
M7	Mir	Few	Low	312	84.5	M (5 + 7), AA, H
M6	Mir	Few	High	436	83.6	M (6 + 8), AA, H
M8	Mir	Few	Low	306	83.5	M (6 + 8), AA, H
O1	Mir	Many	High	217	69.1	AA, H
O2	Mir	Many	High	188	68.6	H
O3	Mir	Many	Low	210	69.4	AA, H
O4	Mir	Many	Low	235	65.0	AA, H
O5	Mir	Few	High	116	70.0	AA, H
O6	Mir	Few	High	191	64.3	AA, H
O7	Mir	Few	Low	119	57.3	AA, H
O8	Mir	Few	Low	212	67.1	AA, H
R1	Reference	Few	High/low	49	78.4	H
R2	Reference	Few	High/low	52	82.3	H
K1	Reference	Few	High/low	67	76.8	H
K2	Reference	Few	High/low	59	79.3	H
C1	Reference	Few	High/low	151	83.0	H
C2	Reference	Few	High/low	161	81.6	H
Y1	Reference	Few	High/low	66	79.3	H
Y2	Reference	Few	High/low	65	78.6	H
M1	Reference	Few	High	718	76.5	M, AA, H
M2	Reference	Few	High	607	76.5	M, H
M3	Reference	Few	Low	462	78.4	M, AA, H
M4	Reference	Few	Low	576	77.2	M, AA, H
O1	Reference	Few	High/low	103	82.4	AA, H
O2	Reference	Few	High/low	106	78.4	AA, H

[a] R, roots; K, crowns; C, culms; Y, young leaves; M, mature leaves; O, old leaves.
[b] AA, amino acids; H, hormones; M, minerals.

differently. Herein, we identify developmental, nutritional and biochemical differences and evaluate various factors that may have contributed to them.

3.1. Leaf and tiller dynamics

We obtained 18 of 37 Super Dwarf plants harvested from the second sowing of the Mir Greenhouse-2 project, 8 and 10 from root modules 1 and 2, respectively. In addition to their parent shoots, these plants produced 89 tillers, and the parent shoots and tillers produced a total of 544 leaves. Numbers of tillers produced per plant varied from 0–12. Tiller frequency (Fig. 2) did not differ across root modules.

Six plants were randomly selected for leaf-area measurements. These had produced 29 shoots (parent shoots plus tillers) and 182 leaves. Numbers of leaves per shoot poorly predicted leaf area per shoot ($r^2 = 0.50$). However, numbers of leaves per plant were good predictors of total leaf area per plant ($r^2 = 0.95$). The difference in r^2 values occurred because most Mir-grown plants possessed both large-leafed and small-leafed tillers. Thus, numbers of leaves per shoot were poor predictors of leaf area per shoot, but numbers of leaves per plant (often possessing small and large-leafed tillers) were good predictors of per-plant leaf area. Because of its uniform occurrence, this large-tiller/small-tiller dimorphism was probably caused by a uniformly-experienced stress, such as high ethylene levels (Levinskikh et al., 2000; Campbell et al., 2001), rather than inconsistently-experienced stresses such as periodic root-zone flooding or drying.

Shoots of root module 1 were longer and the surface areas of their youngest fully-expanded leaves were greater than those of module 2 (Fig. 3). Primary tillers of module 1 were comparable in length to those of the reference plants, but their leaves were narrower, e.g., the mean surface area of the youngest fully-expanded leaves of Mir-grown primary tillers (module 1) was only half the value observed for the reference plants (Fig. 3). Mir-grown plants with many tillers (across modules) were taller than plants with few tillers, but this correlation was weak ($r^2 = 0.17$). Parent shoots of Mir-grown plants produced more tillers than primary or secondary tillers; and parent shoots, primary tillers and secondary tillers tended to be longer than primary, secondary and tertiary tillers, respectively (Fig. 3). This phenomenon suggests either a relaxation of apical dominance (reduced levels of auxins in crowns) or an increase in cytokinin levels in crowns. Evidence for the latter is presented below.

The average number of leaves produced per tiller did not differ among primary, secondary and tertiary tillers of Mir-grown plants

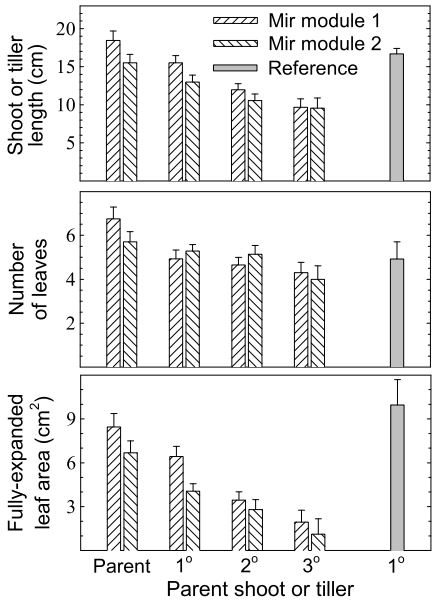

Fig. 3. Mean (± SE) lengths, numbers of leaves, and youngest fully-expanded leaf areas for shoots or tillers of Mir-grown Super Dwarf wheat plants as affected by root module and type of shoot, i.e., parent shoot or primary (1°), secondary (2°) or tertiary (3°) tiller. The shoot type main effect among Mir-grown plants was highly significant ($P \leq 0.001$). The root module main effect was significant ($P \leq 0.05$) for shoot or tiller lengths and for areas of fully expanded leaves. Analyses involving numbers of leaves or interactions (dependent variables) were not significant. Data from primary tillers of reference plants are shown for comparison.

and primary tillers of the reference plants (Fig. 3). However, more leaves formed on parent shoots than on tillers. Also, the mean leaf area of the youngest fully-expanded leaves of parent shoots was greater than that of tillers (Fig. 3). Precocious senescence and death were prevalent among mature leaves and even young leaves of Mir-grown shoots and tillers (Fig. 1B, D). This was not observed among leaves of the reference plants.

Super Dwarf plants grown hydroponically on Earth and exposed to ethylene at 0, 1 and 3 μmol mol^{-1} produced 5.6, 8.0 and 12.6 tillers per plant, respectively (Campbell et al., 2001). This suggests that ethylene aboard Mir (1.1–1.7 μmol mol^{-1}) may have caused the excessive tillering, which is further suggested by pre-flight ground controls. The controls, grown in Balkanine, produced only 2.8 tillers per plant (ethylene largely absent) compared to 7.6 tillers per plant for the 123 d Mir-grown plants (Levinskikh et al., 2000).

Excess NH_4 also increases wheat tillering (Fenn et al., 1995; Camberato and Bock, 1990). 'Apogee' wheat grown in nutrient-enriched clinoptilolite, dolomite and apatite produced many sterile mature and immature tillers compared to hydroponically-grown controls (Steinberg et al., 2000). Both sets had been grown on Earth in the absence of ethylene. The authors attributed increased tillering to NH_4–N, noting that 95% of NH_4 loaded into clinoptilolite had solubilized during plant growth, compared to 21% for K. Levine (1999) also reported increased wheat tillering when plants were grown in NH_4-loaded clinoptilolites. Thus, the excessive tillering observed among the 41 d Mir-grown plants (4.9 per plant) was likely caused by both ethylene and NH_4 (Table 1).

3.2. Tissue water levels

The water content of culms, crowns and live leaves was significantly higher in Mir-grown plants compared to reference plants (Fig. 4). The reference plants had been grown under ideal environmental conditions (Table 1) and compared to Mir-grown plants for the purpose of identifying abnormalities in the latter. A high water content in tissues of Mir-grown plants coupled with an ac-

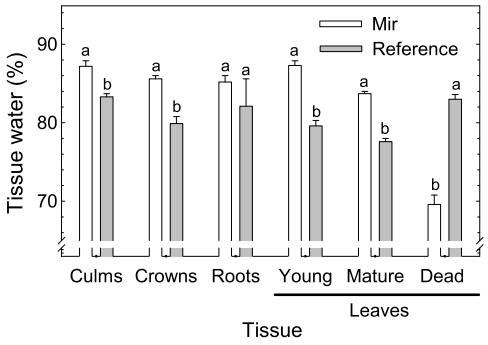

Fig. 4. Mean (± SE) tissue water percentages (FM) for culms, crowns, roots, and young, mature and senescent (dead) leaves of Mir-grown plants and reference plants. Means marked with different letters are significantly different ($P \leq 0.05$) according to T-tests conducted within tissue types ($n = 9$–106).

cumulation of free amino acids typical of those observed in tissues experiencing oxidative stress (discussed below) is consistent with hyperhydricity. In plant tissue cultures, hyperhydricity is caused by constant water availability to roots coupled with high relative humidities in canopies. Both the apoplasm (space between cells) and the cytoplasm are affected. The apoplast water content of hyperhydric tissues can be as high as 85%, with 15% being normal. Excess water in the apoplasm impedes CO_2 and O_2 diffusion, which limits photosynthesis and respiration and leads to an accumulation of reactive oxygen species (ROS) (van den Dries et al., 2013).

In nature, hyperhydricity seldom occurs because root zones and canopies seldom remain saturated. Instead, humid air adjacent to photosynthesizing and transpiring leaves mixes with drier air from the more external environment. This mixing is driven by convective micro-currents of air that form when thin layers of moist air surrounding leaves (boundary layers) are heated by infrared radiation originating from the illuminated leaves. Convection then occurs. The heated boundary-layer air rises and mixes with cooler and drier air, and this reduces boundary layer thickness and promotes transpiration. In weightlessness, convective mixing of moist boundary layer air with external dry air does not occur. Hence, saturated boundary layers increase in thickness. Under such conditions, apoplasmic air spaces, which generally constitute 85 % of the apoplasm (van den Dries et al., 2013), may fill with water. This scenario might explain the high tissue water levels observed in leaves and stems of the Mir-grown plants (Fig. 4) and the lack of stomatal closure at night as measured on the same plants (Monje et al., 2000). Also, absence of stomatal closure at night may have been caused by the super-elevated CO_2 levels to which the plants were exposed (7–10 mmol mol^{-1}, Table 1). Similarly-high CO_2 levels suppressed stomatal closure at night in several dicots (Wheeler, 2006).

Boundary layer thicknesses in weightlessness are theoretically reduced using fans to ventilate leaf surfaces. Based on photosynthesis measurements, such procedures appear to be effective in low to moderate light intensities (Monje et al., 2005). However, 13–16% reductions in whole chain electron transport activity and in the activity of photosystems I and II occurred in weightlessness under high light intensities (Stutte et al., 2005). It remains to be determined whether such reductions are due to weightlessness itself, to ineffectiveness of leaf ventilation procedures in preventing hyperhydricity, or to other causes. Ventilation effectiveness in preventing hyperhydricity in weightlessness could be tested by estimating apoplast volumes of leaves by pycnometry (Raskin, 1983) and by estimating percentages of apoplast volumes filled with water and air using microcentrifugation (Terry and Bonner, 1980; van den Dries et al., 2013).

Harvest and storage procedures for Mir-grown plants may have caused post-harvest water absorption. But this is unlikely because

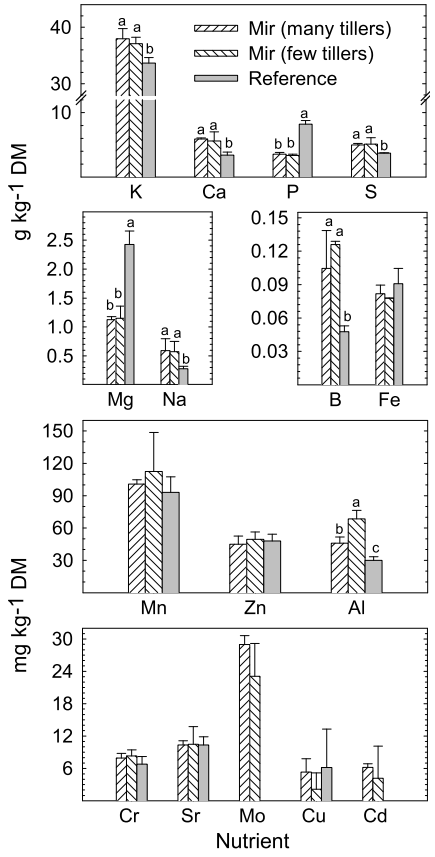

Fig. 5. Mean (± SE) levels of mineral elements for mature leaves of Mir-grown plants and reference plants. Values for Mir-grown plants are averages of six samples (large and small leaves). Values for reference plants are averages across two samples (large leaves only). Mo and Cd levels in reference plant samples were below detectable limits. Means marked with different letters are significantly different ($P \leq 0.05$) according to Tukey multiple comparison tests.

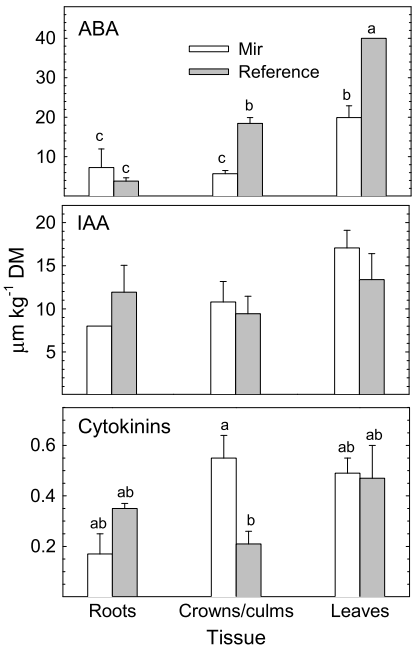

Fig. 6. Mean (±SE) levels of ABA, IAA and cytokinins for roots, leaves, and crowns and culms of Mir-grown plants and reference plants ($n = 6$–10). ABA levels in leaves of reference plants exceeded 40 µmol kg^{-1} DM. Means marked with different letters are significantly different ($P \leq 0.05$) according to Tukey multiple comparison tests.

only live tissues, i.e., culms, crowns, and live leaves, exhibited hyperhydricity. In contrast, the water content of senescent leaves from Mir-grown plants was significantly lower than for senescent leaves of reference plants (Fig. 4). This suggests that hyperhydric conditions, occurring in live leaves of Mir-grown plants, led to anoxia, precocious osmolysis-driven cell death, and rapid desiccation of lysed cells.

3.3. Nutrient and hormone levels

Plants in weightlessness may accumulate K (Nechitailo and Gordeev, 2001; Levine and Krikorian, 2008) and Ca (Kordyum et al., 1984; Belyavskaya, 1996), and our results are consistent with this possibility. Levels of K, Ca, S, Na, B, Al, Mo and Cd were higher in mature leaves of Mir-grown plants compared to those of the reference plants. In contrast, P and Mg levels were lower (Fig. 5). Regardless of these differences, levels of most mineral nutrients were within limits for normal growth (Marschner, 1986), including K (2 to 5 %) and Ca (0.1 to >5.0%), or at least marginally within these limits, including P (low end of 0.3 to 0.5 % range) and S (high end of 0.2 to 0.5 % range). However, Mg levels in Mir-grown leaves were only 20 % of optima (0.1 % vs 0.5 %), and B levels, based on studies conducted with maize (Marschner, 1986), were at potentially toxic levels (>100 mg kg^{-1}).

The nutrient values listed in Table 1 for Balkanine represent potentially-assimilable forms (Ivanova et al., 1997). Actual availability depends on dissolution and ion exchange rates, which vary among clinoptilolites based on original nutrient content (Allen et al., 1993, 1995; Levine, 1999; Steinberg et al., 2000). Nevertheless, the observed high tissue levels of certain minerals in leaves of the Mir-grown plants, e.g., K, Ca, Na and B (Fig. 5), were likely caused by high levels of availability in the liquid component of the Balkanine substrate.

Because of small sample sizes and few replications (Table 2), differences in hormone levels among specific plant tissues were not detected. However, differences were detected when values from adjoining tissues were grouped together. Most prominent were high ABA levels in crowns, culms and leaves of reference plants compared to Mir-grown plants (Fig. 6). The reference plants may have experienced osmotic stress, which causes ABA levels to rise (Danquah et al., 2014). It is also possible that high levels of ethylene aboard Mir suppressed ABA synthesis in the Mir-grown plants.

As a gaseous plant hormone, ethylene participates in regulating many physiological and developmental processes (Abeles et al., 1992). Plant sensitivities to ethylene range widely depending on genotype (Klassen and Bugbee, 2002; Hays et al., 2007). Highly sensitive plants show responses at 0.2 nmol mol^{-1}, but resistant plants might not show responses until levels approach 1.0 mmol mol^{-1} (Merchante et al., 2013). Ethylene levels aboard Mir were well within this range of sensitivity and varied from 1.1–1.7 µmol mol^{-1} (James et al., 1997; Bingham et al., 1996a). Ethylene inhibits ABA synthesis and accumulation (Wilkinson et al., 2012) and adversely affects ABA sensitivity (Wilkinson and Davies, 2010). Hence, ethylene inhibition of ABA synthesis likely contributed to the low levels of ABA observed in the crowns, culms and leaves of the Mir-grown plants (Fig. 6).

In subsequent experiments, separate sets of Super Dwarf wheat plants grown on Earth were exposed to ethylene levels ranging from 0–20 µmol mol^{-1}. Results from these studies indicate that exogenous ethylene aboard Mir likely caused seed abortion in plants from the first sowing and stunted growth and excessive tillering in plants from both sowings (Campbell et al., 2001; Bubenheim et al., 2003; Salisbury et al., 2003).

Levels of CO_2 aboard Mir were also above healthy levels, and ranged from 7–10 mmol mol^{-1} (Monje et al., 2000), or about 18 to 25-fold higher than on Earth. In ground-based studies,

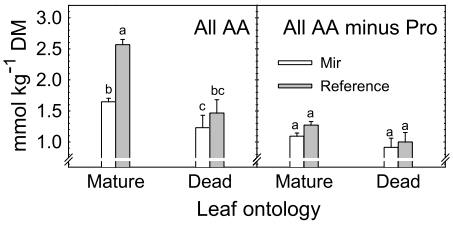

Fig. 7. Mean (±SE) levels of all amino acids (AA) combined and all AA combined minus proline (Pro) for mature and senescent (dead) leaves of Mir-grown plants and reference plants. Means were obtained from 6–8 samples of leaves from Mir-grown plants and from 2–4 samples of leaves from reference plants. Means marked with different letters are significantly different ($P \leq 0.05$) according to Tukey multiple comparison tests.

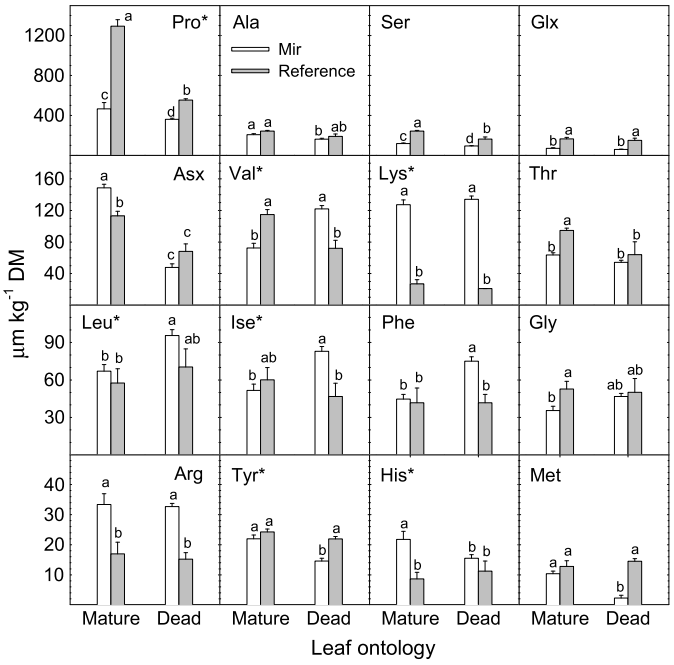

Fig. 8. Mean (± SE) levels of amino acids for mature and senescent (dead) leaves of Mir-grown plants and reference plants. Means were obtained from 6–8 samples of leaves from Mir-grown plants and 2–4 samples of leaves from reference plants. Means marked with different letters are significantly different ($P \leq 0.05$) according to Tukey multiple comparison tests. Starred amino acids are those known to accumulate during oxidative stress (Xiong and Sheen, 2014). Pro, proline; Ala, alanine; Ser, serine; Glx, glutamine and glutamic acid; Asx, asparagine and aspartic acid; Val, valine; Lys, lysine; Thr, threonine; Leu, leucine; Ise, isoleucine; Phe, phenylalanine; Gly, glycine; Arg, arginine; Tyr, tyrosine; His, histidine; Met, methionine.

wheat yields were higher when CO_2 levels were enriched 3-fold; but when CO_2 levels were elevated by 8-fold or more (up to 20 mmol mol^{-1}), endogenous ethylene synthesis increased and grain yields fell (Bugbee et al., 1994; Grotenhuis and Bugbee, 1997; Jiang et al., 1998).

Cytokinin levels in crowns and culms also differed with levels being higher in Mir-grown plants (Fig. 6). Cytokinins, in a poorly-understood interplay with auxins and other molecules, promote the precocious activation of axillary buds (Vanstraelen and Benkova, 2012). ABA limits cytokinin synthesis and inhibits its export from roots and its accumulation in shoots (Nishiyama et al., 2011; Ha et al., 2012). Hence, the excessive tillering in Mir-grown plants and in ground experiments exposed to high ethylene levels (Campbell et al., 2001; Bubenheim et al., 2003) may have involved a three step process: ethylene inhibition of ABA synthesis, increased cytokinin synthesis in roots and its transport to crowns and culms (in the absence of ABA), and cytokinin-induced precocious tiller-bud activation in crowns. Ethylene also causes newly initiated leaves to elongate rapidly (Hunter et al., 1999), which may explain the formation of many long and narrow leaves among the unusually-small tillers of the Mir-grown plants.

Summed together, levels of free amino acids were higher in the mature leaves of healthy reference plants compared to mature leaves of Mir-grown plants, but no differences in summed totals were observed for senescent leaves (Fig. 7). When individual amino acids were considered independently, we observed that levels of proline, alanine, serine, glutamine plus glutamic acid, threonine, glycine, tyrosine and methionine were higher in the leaves of reference plants (Fig. 8). Levels of these amino acids, except for glutamine plus glutamic acid and glycine, were lower in senescent leaves. This is consistent with processes of nutrient-export from cells, which generally accompany leaf senescence (van Doorn et al., 2011).

When proline levels were ignored, total amino acid levels in mature leaves did not differ between Mir-grown plants and reference plants (Fig. 7). Proline was the most abundant amino acid, and its levels were higher in mature leaves of the reference plants (Fig. 8). When plants experience drought, lysine is converted to glutamic acid, glutamic acid is converted to proline, and proline accumulates to osmotically-active levels (Galili et al., 2001; Min et al., 2014). Depleted lysine levels, elevated levels of glutamine plus glutamic acid, elevated levels of proline (Fig. 8), and low tissue water levels (Fig. 4) in leaves of the reference plants suggest that these leaves were osmotically stressed compared to leaves of the Mir-grown plants.

TARGET OF RAPAMYCIN (*TOR*) encodes a highly conserved serine/threonine protein kinase that regulates multiple gene networks involved in nutrient, energy and stress signaling (Baena-Gonzalez, 2010; Ren et al., 2012; Robaglia et al., 2012; Xiong and Sheen, 2014). Normally, TOR promotes ribosome and protein synthesis while preventing autophagy and plant cell death (Ahn et al., 2011; Williams et al., 2014). However, ROS, which accumulate during energy starvation, hypoxia and other stresses, suppress synthesis and functioning of TOR. Ribosome and protein synthesis are then curtailed, autophagy (protein catabolism) occurs, catabolism-resistant free amino acids accumulate, including branched-chain (leucine, isoleucine and valine), aromatic (tyrosine and tryptophan) and other (lysine, histidine and proline) amino acids (Xiong and Sheen, 2014), and programmed cell death ensues. Interestingly, amino acids consistent with such ROS-induced autophagy, including leucine, isoleucine, valine, lysine and histidine, accumulated to high levels in mature and senescent leaves of the Mir-grown plants (Fig. 8). These accumulations coupled with hyperhydricity (Fig. 4) and precocious leaf senescence (Fig. 1B, D) suggest that inadequate leaf ventilation caused leaf hyperhydricity followed by hypoxia, oxidative stress, TOR suppression, autophagy and cell death. Ventilation (1500 L min^{-1}) through Svet was not interrupted following the gas-exchange experiments (Monje et al., 2000), i.e., during the last 16 d of the 41 d growth period. But tunneling of air flow through the canopy may have occurred, the result being that many leaf surfaces may not have been well ventilated.

Oxidative stress was recently documented in weightlessness-grown plants of barley (Shagimardanova et al., 2010) and *Brassica* (Sugimoto et al., 2014). In both cases, the plants were thought to have been well ventilated, and the oxidative stress was attributed to weightlessness. However, our results suggest that reductions in boundary layer moisture by leaf ventilation in weightlessness may be less effective, or more difficult to achieve, than previously thought. The Svet greenhouse in our study moved 1500 L of air through the canopy per minute, yet evidence of chronic leaf hyperhydricity was observed. We suggest that hyperhydricity of leaves be measured in future weightlessness experiments.

3.4. Stresses unique to weightlessness versus weightlessness-aggravated terrestrial stresses

A confluence of the signal transduction pathways of diverse biotic and abiotic stresses occurs upstream of the oxidative stress pathway, the latter being a central coordinator of various nutrient, energy and stress signaling networks (Xiong and Sheen, 2014). Due to this upstream confluence, plant responses to diverse stresses are often similar, and this may include weightlessness-specific stresses. Similarities in stress responses complicate cause-and-effect analyses (Fujita et al., 2006; De Micco et al., 2014). In the past, we speculated that the developmental anomalies observed in the present study, and those observed among plants of the first sowing (Salisbury, 1997; Levinskikh et al., 2000; Campbell et al., 2001; Salisbury et al., 2003), were caused exclusively by high ethylene levels aboard Mir. But such speculation is not entirely consistent with current views.

Even when growth conditions are optimized, weightlessness is now expected to extract a toll on plant performance. A major goal for future space biology experimentation will be to understand the extent of this toll, and this will require additional physiological, developmental and molecular studies. Properly designed, these studies will differentiate direct from indirect effects of weightlessness on plant performance, and they will also begin to define ideal weightlessness-tolerant plant prototypes (De Micco et al., 2014). Such prototypes may ultimately be achieved by plant breeding and genetic engineering, but knowledge of gravitropism, phototropism and other molecular and physiological processes affected by weightlessness will be central to such germplasm improvement.

Though details remain vague, gravitropism is initiated by sedimentation processes in shoot endoderm and root columella cells, the sedimentation process itself representing the gravitropic signal (Hashiguchi et al., 2013; Strohm et al., 2014). This signal is followed by movement of auxin to the underside of shoot and root cells. Curvature then occurs such that shoots and roots grow parallel to the gravity vector. Auxin influx and efflux transporters are central to this up or down growth (Geisler et al., 2014; Ueda et al., 2014), and the process itself is fine-tuned by auxin sensitivity (Salisbury, 1993). Auxin transporters also are central to phototropic growth, i.e., of shoots toward light and of roots away from light. Instead of gravity-dependent sedimentation, phototropic signals are generated by photoreceptor kinases, primarily phototropin 1 and 2, that are autophosphorylated upon blue light irradiation (Christie and Murphy, 2013). Recent studies indicate that gravitropism is attenuated by strigolactone hormones to effectuate angular growth. The mode of action involves local reductions in auxin biosynthesis (Sang et al., 2014).

Essentially all phases of the life cycle involve auxin-mediated processes (Vanstraelen and Benkova, 2012; Ceccato et al., 2013). Since gravity fine-tunes inter and intracellular auxin transport, it is reasonable to suspect that weightlessness may negatively impact many plant development processes. Whether auxins are involved or not, evidence suggests that weightlessness adversely affects cell cycle regulation (Matía et al., 2005, 2010), cell wall deposition and elongation (de Micco et al., 2008), and possibly the regulation of ROS formation and catabolism (Sugimoto et al., 2014).

4. Conclusion

Evidence of excessive oxidative stress in leaves of Mir-grown wheat is provided herein. This may have been caused by high ethylene levels aboard Mir or even by weightlessness itself. However, our data suggest another explanation. Leaves of the Mir-grown plants contained excessive levels of water, a phenomenon consistent with leaf hyperhydricity in plant tissue cultures on Earth. Additionally, these leaves contained free amino acid profiles consistent with oxidative stress and subsequent autophagy, and they tended to senesce precociously. These findings suggest that the leaf ventilation provided by the Svet greenhouse aboard Mir may have failed to remove sufficient quantities of boundary-layer water. Since oxidative stress is frequently observed in tissues of plants grown in weightlessness, ineffective leaf ventilation as a possible cause should be considered.

On Earth, water is removed from cell surfaces of leaves at the molecular level based on heat-driven micro-convective air currents that originate immediately adjacent to irradiation-warmed cell surfaces. In contrast, removal of water from cell surfaces by forced-air ventilation in weightlessness depends, at least at the boundary layer level, on diffusion of water through air, which may be a much less effective process. Before we can determine if weightlessness itself causes oxidative stress, it will be necessary to rule out hyperhydricity, which may present as a progressive syndrome ranging, for example, from 15 % of apoplast free space being filled with water to as high as 85 %. Even mild but chronic hyperhydricity may cause leaf tissue hypoxia and ROS formation. Fortunately, hyperhydricity is detected by simple procedures that measure percentage water content in the apoplast free spaces of leaves. Such tests might reveal that crops with high density canopies, such as cereals, may be less suited to spaceflight production, or they may require either growth in lower relative humidity environments or more vigorous and possibly not as yet developed water removal systems. Testing for hyperhydricity among weightlessness-grown plants may also reveal opportunities to breed or engineer leaf epidermal surfaces such that a more conducive exchange of water vapor occurs at cell surfaces under weightlessness conditions.

Acknowledgements

We acknowledge technical support provided by William Campbell, Rubin Nan, and Becky Kowallis, USU, technical and administrative support provided by Gary Jahns, Kristina Lagel, Sally Greenawalt and Terry Schnepp, NASA Ames Research Center, and flight support provided by Vladimir Sychev, Margarita Levinskikh and Igor Podolsky, Russian Academy of Sciences, Institute of Biomedical Problems, Moscow, Russia. This research was supported by NASA Grant NCC 2-831 and by the Utah Agricultural Experiment Station, Project 426, Utah State University, Logan UT 84322-4810 (approved as UAES journal paper number 8749).

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